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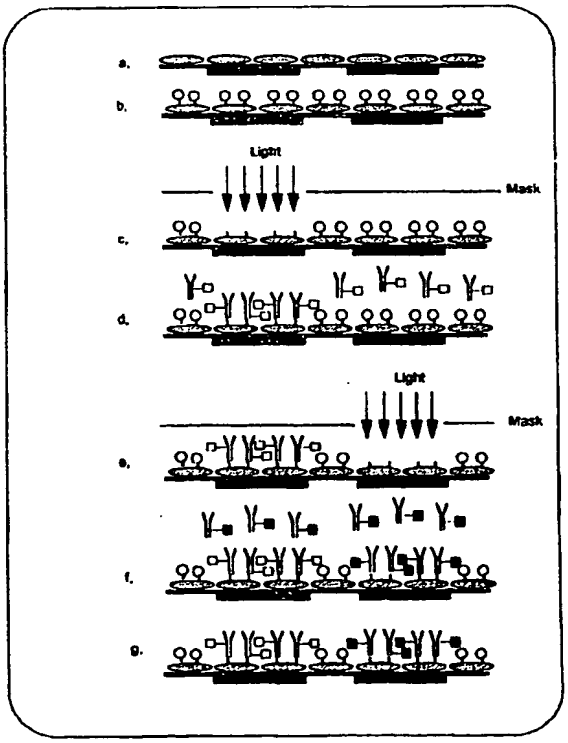
(57) Abstract

There is described a device which has a surface coated with a biomolecule in a pre-determined pattern. The molecule is attached to the surface via a photosensitive binding moiety itself attached to the surface via a linking moiety. Preferably the linking moiety is avidin or a derivative thereof and the photosensitive binding moiety is photobiotin or a derivative thereof. The pattern of binding of the biomolecule is determined by the selective irradiation of non-irradiation of the photosensitive binding moiety. In a preferred embodiment two or more different ligands are bound to the surface in a pre-determined pattern. The device according to the invention may be of use in multi-analyte sensors in molecular electronics, in directional propagation of cell growth and in altering the behaviour of cells. The device may also be used to bind nucleotides which are subsequently manipulated or used as a probe or template.



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"SURFACE-PATTERNED DEVICE"

The present invention relates to a device wherein molecules are attached to a surface in a pre-determined pattern. A process for producing such a device is also disclosed.

Various applications in technology require a device to have a surface which is coated with a molecule, such as an organic molecule. Generally, a uniform layer of the molecule required is bound to the surface. Optionally the bound molecule may then be used to attach other molecules to the surface.

Surfaces coated with biomolecules in this way have many applications, for example in assays or diagnostic tests. One popular assay is an immunoassay, involving the use of antibodies to selectively bind to an antigen of interest. Frequently, the antibody may be bound to a surface giving a convenient diagnostic device. Other applications where binding biomolecules to a surface is useful includes the separation and purification of biomolecules.

GB-A-2141544 discloses a method of binding biomolecules to a surface in a particular pattern. The biomolecules are bound via a photosensitive intermediate organic molecule, such as N-(4-azido-2-nitrophenyl)-1,3-diaminopropane. By using a mask, the photactivatable organic molecule is light activated in specific areas only and the biomolecule is subsequently only able to bind to those areas.

The process of GB-A-2141544 may result in non-specific binding, since biomolecules other than the one of interest may also be bound to the activated photosensitive intermediate resulting in a poor quality product. Problems in binding the molecules of interest may also occur due to steric restrictions. Further, the process described in GB-A-2141544 is dependent upon covalent attachment of the photosensitive intermediate organic molecule to the surface.

The present invention seeks to overcome the problems encountered in the prior art and to provide patterning of molecules upon a surface in a precise manner.

In one aspect, the present invention provides a device having a surface, said surface having a ligand bound thereto in a pre-determined pattern, the binding of said ligand being determined by the irradiation or non-irradiation of a photosensitive binding moiety attached to said surface via a linking moiety.

The coated surface of the device is preferably capable of producing measurable change. The change may be detected by any suitable means, for example optically, spectrophotometrically, piezoelectrically, calorimetrically or by measuring magnetic field strength.

Desirably, the device of the present invention has a surface on which at least two different ligands are arranged thereon in a pre-determined manner.

The linking moiety must be able to be uniformly bound to the surface of interest. Selection of the technique to bind the linking moiety may thus depend upon the chemical character of the surface. Furthermore the linking moiety preferably has the function of preventing or reducing non-specific binding. The linking moiety may also be of utility in spacing out the binding moiety to avoid steric hindrance problems in binding the ligand. Preferably the linking moiety may be orientated in a particular manner on the surface.

The term "functional equivalent" is used herein to refer to any modified version of a moiety which retains the basic function of the moiety in its unmodified form. As an example, it is well-known that certain alterations in amino acid or nucleic acid sequences may not affect the protein encoded by that molecule or the function of the protein. It is also possible for deleted versions of a molecule to perform a particular function as well as the original molecule.
Even where an alteration does affect whether and to what degree a particular function is performed, such altered molecules are included within the term "functional equivalent" provided that where the function concerned is required for production of the device according to the invention then this function is performed sufficiently to render the device operational within the degree of accuracy required for the ultimate end use of the device.

Conveniently, the linking moiety is itself an organic molecule. The linking moiety may be a macromolecule, for example a macromolecule having a molecular weight of at least 500Da, or the linking moiety may be a biomolecule such as polypeptides or proteins, mono-, di- or poly-saccharides, or functional equivalents thereof. However, non-biological molecules are not excluded and examples include polymers and other organic molecules. Preferably, the linking moiety is a polypeptide or protein, and particularly preferred examples include avidin, streptavidin or functional equivalents thereof.

The linking moiety may be bound to the surface by any type of association, including non-covalent and covalent binding, ionic interaction and intermolecular associations such as hydrogen bonding, and Van der Waals attractions. Non-covalent interactions may be preferred in certain applications.

Alternatively, the linking moiety may be attached to the surface by physical entrapment.

It may be desirable in certain applications to attach the linking moiety to the surface so that substantially all of the linking moieties are orientated in the same or similar direction on at least part of the surface.

It is not necessary for the linking moiety to be directly attached to the surface, and in some circumstances the surface may be coated (optionally several times) before the linking moiety is attached to a layer thereof, usually the uppermost layer. Where the linking moiety is attached to the surface by entrapment in a carrier substance, it may be desirable

to coat the surface with an admixture of linking moiety in the carrier substance, the carrier adhering to the surface and physically entrapping the linking moiety.

The binding moiety may be any photosensitive entity which binds to said linking moiety. As an example, the binding moiety may be based on a biomolecule, such as a protein, polypeptide, mono-, di- or poly-saccharide, polynucleic acid and the like, or functional equivalents thereof. Also suitable as the binding moiety are small biological or non-biological molecules such as a photosensitive derivatives of biotin (2-keto-3,4-imidazolido-2-tetrahydrothiophen-n-valeric acid). A suitable photosensitive derivative of biotin is the molecule (N-(4-azido-2-nitrophenyl)-N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine), commonly known as "photobiotin". Alternatively, the binding moiety may be any protein or polypeptide (or functional equivalent thereof) able to bind to the specific ligand of interest. In this regard, mention may be made of enzymes and antibodies which are suitable for use as said binding moiety. In particular, photosensitive antibodies (for example monoclonal antibodies) or biotin are preferred.

It is essential that the binding moiety is photosensitive, that is to say that the binding moiety is sensitive to irradiation. The term "photosensitive" is used herein to indicate that the binding moiety is altered (physically and/or chemically) by exposure to electro-magnetic radiation. Preferably, the binding moiety is activated by electro-magnetic irradiation. The binding moiety may be irradiated by any type of light including visible light, UV light and infra-red light.

Generally, irradiation of said binding moiety occurs in pre-selected areas to impose the desired pattern thereon. Selective irradiation may be achieved by any known method, but one convenient way is to superimpose a mask or screen of irradiation-absorbing or reflecting material over the surface. The shape of the mask is transferred into the surface by the alteration of binding moieties exposed to the radiation. Other means of selectively altering binding moieties include the use of focused radiation or irradiation sources such as lasers.

In one embodiment, irradiation causes activation of the photosensitive binding moieties exposed to the radiation. Only the activated binding moieties are able to bind to the ligand. In this embodiment the pattern of ligand binding corresponds to those areas exposed to irradiation.

In another embodiment, irradiation alters the exposed binding moieties. Only the binding moieties which have not been altered by such exposure (that is, only the binding moieties which were not irradiated and which retain their original configuration), are able to bind to the ligand. In this embodiment the pattern of irradiation corresponds to areas not bound by ligand.

The binding moiety will usually be in its photosensitive form when initially contacted with the linking moiety. However this is not essential and under certain circumstances it may be more convenient to photosensitize a form of the binding moiety <u>in situ</u> after attachment to the linking moiety has taken place.

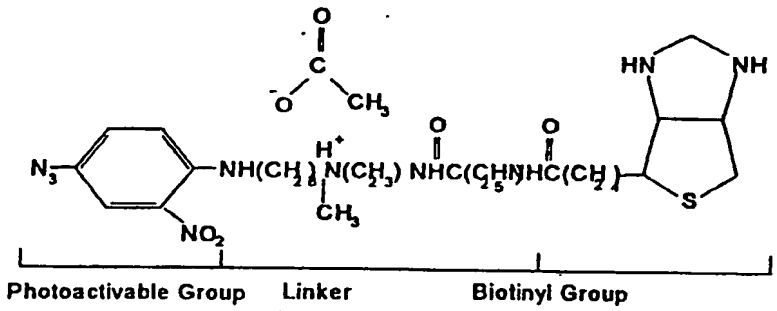
The ligand can be any molecule, including proteins,

polypeptides, electron mediators, amino acids, sugars, polysaccharides, nucleic acids and other organic or inorganic molecule (and functional equivalents thereof). The ligand may itself be able to bind to a further moiety. For example, the ligand may be an antibody (especially a monoclonal antibody) which may be bound via its Fc region to the binding moiety. Conveniently, the ligand may be a difunctional antibody (especially a monoclonal antibody), that is an antibody having the ability to bind two different haptens separately. Alternatively, the ligand may be an enzyme (or a functional equivalent thereof) or a polynucleic acid.

In a further embodiment, it is possible to bind two or more different ligands onto the binding moiety in distinct areas. This can be achieved, for example, by irradiation of the surface only in those areas where the first ligand is to be bound. The first ligand is then brought into contact with the irradiated surface, allowed to bind thereto and any excess ligand washed off. The surface may then be selectively exposed to radiation a second time, once the first ligand has bound, thus activating a second selection of binding moieties. A second ligand may be bound to the binding moieties so activated. This process may be repeated as many times as required for each set of ligands to be selectively bound to the surface in a pre-determined way.

It is possible to coat the binding moieties not reacted to ligand by use of a blocking moiety. Suitable blocking proteins are known in the art, but mention may be made of milk proteins such as casein, TRIS™ buffer, or serum albumins such as HSA or BSA.

In a preferred embodiment, the linking moiety may be the tetrameric proteins avidin, streptavidin, functional equivalents or mixtures thereof. Certain avidins and streptavidins have low non-specific binding properties thus eliminating non-specific adsorption. Any other protein or polypeptide with this characteristic will be suitable as a linking moiety in the present invention. It is especially preferred if the binding moiety used therewith is a photosensitive analogue of biotin (vitamin H) which binds to avidin and streptavidin with an association constant of $10^{15}M^{-1}$. The photosensitive analogue of biotin may be photobiotin (ie N-(4-azido-2-nitrophenyl)-N'(N-d-biotinyl-3-aminopropyl-N'-methyl-1,3-propanediamine). Photobiotin contains an arylazide group which is stable in the dark, but upon exposure to ultra-violet or blue light (having a wavelength of 340-375nm) generates highly reactive aryl nitrene group which may bind other molecules. The photobiotin may incorporate a spacer moiety to reduce steric hindrance on binding the ligand. The structure of the spacer-photobiotin molecule is shown in Formula 1 below:



The surface to be coated may be any convenient type, including silicon, silicon nitride, silicon dioxide, glass, quartz, metals, metal oxides, polymers including nitrocellulose and nylon, and mixtures thereof. Preferably, the surface is gold, platinum, silicon or silicon oxide, dioxide or nitride and mixtures thereof.

In a preferred embodiment, the present invention provides a device having a surface, said surface having a ligand arranged thereon in a pre-determined pattern, the binding of said ligand being determined by the irradiation of a photosensitive biotin binding moiety attached to the surface via an avidin linking moiety. The ligand may desirably be an enzyme, for example glucose oxidase, an immunoglobulin, for example an antibody, or a hormone, for example human gonadotrophins.

The precise binding of a ligand in a pre-determined pattern has many applications. One particularly promising aspect is the use of a surface according to the invention as part of a multi-analyte sensor, in particular a multi-analyte immunosensor. The present invention is particularly suited to this application since each ligand type can be located on the surface with accuracy, eliminating "cross-talk" in the sensor.

There has been considerable interest over the last decade in the development of amperometric immunoassay, primarily as the technique has the potential to combine the advantages of using a sensitive enzyme label with a convenient and safe format (see Frew et al, Anal Chem 59: 933A-944A (1987)). Although there is now an extensive literature in the development of such assays for both clinical and environmental analysis (see

"Biosensors", Hall, Wiley (1990)), to date there has been no published description of the fabrication of a true multi-analyte amperometric biosensor, in which more than one high molecular weight species is measured simultaneously by a single device. Of the existing multi-analyte immunosensors, commercial devices that have been produced are qualitative optical assays (based upon agglutination) for low molecular weight analytes (eg the Triage™ and Advisor™ systems (see Buechler et al, Clin Chem 38: 1678-1684; and Parsons et al Clin Chem 39: 1899-1903 (1993)) for detecting drugs of abuse). Such systems are unsuitable for quantitative analysis.

The use of simultaneous multi-analyte immunoassay is required in a number of clinical situations including the measurement of hormones related with thyroid function and the measurement of gonadotrophins for the investigation of infertility. An example where such an assay would be useful is for the measurement of follicle stimulating hormone (FSH) and luteinising hormone (LH), which can be used as a "fertility test" in women, or to differentiate between primary and secondary hypogonadism. FSH and LH are both glycoprotein hormones, with relative molecular masses of approximately 34,000 and 28,500 respectively. Circulating gonadotrophin concentrations are widely monitored in diagnosis and treatment of infertility, as well as in developmental disorders. For example, in primary hypogonadism, the concentrations of gonadotrophins increase in a process controlled by negative feedback, whereas in secondary hypogonadism low levels of FSH and LH are the cause of the disorder. A particular situation where the use of an immunosensor for the measurement of gonadotrophins is likely to be

beneficial, is for *in vitro* fertilisation procedures where the rapid measurement of gonadotrophins is important.

The major challenge for designing a multi-analyte immunosensor is in developing a technique for patterning of antibodies at discrete transducer sites, ie a method which enables immunologically active IgG to be selectively positioned at particular sites whilst avoiding problems associated with non-specific binding at other sites. Previously a number of methods for immobilising antibodies in such "patterns" on a surface have been reported, although their potential applicability to biosensor technology has been limited by the number of functional proteins that can be patterned and/or by non-specific binding of protein to undesignated areas of the sensor or its surround (see Britland et al, Biotechnol. Prog. 8: 155-160 (1992); Bhatia et al, Anal Biochem, 208: 197-205 (1993); Connolly, Trends in Biotechnology 12: 123-127 (1994)).

The device according to the invention may also be used to selectively deposit molecules onto a surface in ordered arrays for use in molecular electronics. Thus, groups of molecules may be positioned precisely with respect to other groups of molecules, or to electronic structures in order to build up functional molecular architectures. As is the case for the design of a diagnostic device, a variety of different transducer materials may be used as immobilisation substrates, and the design of arrays that do not exhibit cross-talk is of great importance. Such devices could be used for design of bioelectronic memory cells, or more elaborately, in biological computing.

A further application of this invention is in a device for the directional propagation of an individual cell, (eg nerve cells or their neurites) on a patterned substrate. In this case, the essential prerequisites are the same as for the design of a diagnostic device, in so much as it is desirable to position organic, inorganic or biological molecules (eg nerve growth factor) onto a pre-defined substrate with no interference from non-specific adsorption. The patterned molecules will act as a chemotactic or topological template for guidance of the cell, which will grow preferentially in a given direction determined by the pattern. Particularly important applications are the manufacture of devices either to control endothelial cell growth for wound healing, or to control nerve cell growth to promote regeneration.

In addition, it may be desirable to use a patterning technique to alter the behaviour of many cells. For example, by coating appropriate molecules onto a surface, it will be possible to differentially promote or prevent cell growth on the outer surface of a miniature sensor in order to enhance the biocompatibility properties of the device.

The device according to the present invention is also of utility as a matrix for binding nucleotides, for example DNA or RNA molecules. The nucleotides may be single stranded or double stranded. The nucleotide bound to the device may be used as a probe (for example for nucleotides having a complementary sequence or to bind nucleic acid binding proteins) or may be manipulated by chemical reactions or by genetic engineering techniques. A nucleotide bound to the device according to the invention may be used as a

template in a polymerase chain reaction (PCR) method.

In a further aspect, the present invention provides a process for forming a surface having a ligand bound thereto in a pre-determined pattern, said process comprising the following steps:

i) binding a linking moiety to a coated or uncoated surface;

ii) binding a photosensitive binding moiety to said linking moiety;

iii) selectively exposing said photosensitive binding moiety to irradiation in a pre-determined pattern;

iv) exposing said binding moiety to said ligand and allowing the ligand to bind to said binding moiety in accordance with the irradiation exposure;

v) optionally removing excess ligand by washing;

vi) optionally exposing said ligand to a further molecule capable of binding thereto; and

vii) optionally repeating steps iii) and iv) with a different ligand.

Figures 1 to 3 are schematic representations of the process of the present invention.

Figures 4 to 7 are graphs showing the results of Example 7.

Figure 4 is a graph showing the electrochemical

response (nA) of the disposable multi-analyte sensor to FSH (Ul^{-1}) in buffer. There is a linear response at the FSH electrode (o) to FSH over the concentration range 0-100 Ul^{-1} and there is minimal response at the LH electrode (•) to FSH, indicating low non-specific binding.

Figure 5 is a graph showing the electrochemical response (nA) of the disposable multi-analyte sensor to LH (Ul^{-1}) in buffer. There is a linear response at the LH electrode (•) to LH over the concentration range 0-100 Ul^{-1} and there is minimal response at the FSH electrode (o) to LH.

Figure 6 is a graph showing results for FSH in serum, obtained using the multianalyte immunosensor (as described in Example 7), plotted against those obtained using an established DELFIA technique. Each sample was measured in triplicate and the error bars represent two standard deviations around the mean.

Figure 7 is a graph showing results for LH in serum, obtained using the multianalyte immunosensor (as described in Example 7), plotted against those obtained using an established DELFIA technique. Each sample was measured in triplicate and the error bars represent two standard deviations around the mean.

A diagrammatic representation of an example of the process according to the invention is shown in Figures 1 and 2 and demonstrates the patterning of three species using avidin as the linking moiety and photobiotin as the binding moiety, and exposing defined areas of the surface to light by the use of a mask.

Initially, avidin is coated over the entire surface (Step a), photobiotin is then added and binds to the avidin (Step b). Exposure of selected areas to light results in cleavage of the photobiotin molecule (Step c), and when the first ligand to be immobilised is added, specific immobilisation occurs due to reaction with the exposed aryl nitrene group of the cleaved photobiotin.

After washing off any unbound material, the procedure is repeated with the second ligand to be immobilised (Steps e to g). Again any unbound material is washed off, and the entire surface is then exposed to light (Step h), and a blocking species may be added whose function is to bind to all of the previously unoccupied photobiotin molecules and so block further reactions involving the photobiotin molecule (Step i). Any excess of this blocking species is washed off leaving the surface with the desired pattern of molecules on its surface (Step j).

Figure 3 is a schematic representation of the immobilisation procedure: (a) Avidin with photobiotin immobilised onto a surface; (b) exposure of selected areas to light through a mask results in activation of the photobiotin molecule, specifically immobilising any protein in the solution; (c) unbound material is removed by washing, and the procedure repeated with a second protein; (d) the entire surface is exposed to light, and a blocking molecule bound to all unreacted photobiotin groups.

The invention will now be further illustrated by the following, non-limiting examples:

Example 1

(1) Light dependent coupling of glucose oxidase to a gold surface.

Avidin D™ (Vector Products Ltd, USA) was immobilised on to two identical gold electrodes by placing the electrodes in 5ml of a 0.2mg ml^{-1} solution of Avidin D in phosphate buffered saline pH 7.4 (PBS) for one hour at ambient temperature. After extensive rinsing with PBS the electrodes were then incubated in 5ml of a 10µg ml^{-1} solution of long arm photobiotin in PBS for 20 minutes under dark room conditions. After extensive rinsings with PBS each electrode had 50µl of identical solutions of glucose oxidase in PBS placed onto it, one electrode was retained in dark room conditions whilst the other was exposed to light from a high pressure mercury vapour lamp for 15 minutes. After extensively rinsing both electrodes with PBS under dark conditions, 50µl of a 10 mg ml^{-1} solution of bovine serum albumin in PBS was added to each electrode and they were exposed to light from a high pressure mercury vapour lamp for 15 minutes.

(2) Assay for glucose oxidase activity

An amperometric assay was performed using the modified gold surface as a working electrode, with a Ag/AgCl electrode as a reference and a bare platinum flag as a counter electrode. Chronoamperometry was performed in working solutions containing 0 mM and 100 mM glucose solutions in 15mls PBS. The solutions also contained 25 mM KCl as the electrolyte. Initially, the working electrode was poised at a potential of 0V for 300 seconds after which the potential was stepped to

650mV for 120 seconds during which time the current was monitored.

	Current 30 seconds after application of 650 mV potential μA	
	Electrode exposed to light	Electrode kept in dark
0 mM glucose	0.086	0.089
100 mM glucose	0.358	0.084

Example 2

(1) Light dependent coupling of an antibody to a gold surface

Avidin D and photobiotin were immobilised onto two gold electrodes according to the process of Example 1. After extensive rinsing with PBS each electrode had 50μl of identical solutions of rabbit anti-rat IgG in PBS placed onto it and they were exposed to light from a high pressure mercury vapour lamp for 15 minutes.

(2) Assay for antibody activity

One of the electrodes was placed in 5 ml of 10μg ml^{-1} rat IgG in PBS for 60 minutes, whilst the other was placed in 5 ml of 10μg ml^{-1} rabbit IgG for 60 minutes. Following this both electrodes were extensively washed with PBS and were incubated in 5 ml of a solution of 20μg ml^{-1} horseradish peroxidase labelled rabbit anti-rat IgG ambient temperature for 60 minutes. An amperometric assay was performed using the modified

gold surface as a working electrode, with a Ag/AgCl electrode as the reference and a bare platinum flag as a counter electrode. After thorough rinsing the electrodes were placed in 15ml of 25mM KCL, 10mM hydrogen peroxide, 1mM ferrocene monocarboxylic acid and a potential of 0V was applied for 10 seconds followed by 320 mV for 120 seconds during which time the current was monitored.

Sample	Current 30 seconds after application of a 320 mV potential μA
Electrode 1 in PBS	0.079
Electrode 2 in PBS	0.083
Electrode 1 + Rat IgG	0.243
Electrode 2 + Rabbit IgG	0.084

Example 3

(1) Light dependent coupling of a protein to a silicon oxide surface

A wafer of silicon dioxide was immersed in a 1% solution of 1,3-trimethoxysilylpropylethylene diamine in 95% ethanol 5% distilled water for 120 seconds. After removing the wafer from this solution it was rinsed briefly in 95% ethanol 5% distilled water before being heated at 120°C for 30 minutes. The wafer was immersed in a 2% solution of gluteraldehyde in PBS for 15 minutes, and then in a solution of 40mM sodium cyanoborohydride, containing 0.2mg ml^{-1} Avidin D in PBS for 30 minutes at ambient temperature. After extensive rinsing with PBS the wafer was then incubated in 5ml of a 10μg ml^{-1} solution of long arm photobiotin in PBS for

20 minutes under dark room conditions. After extensive rinsing with PBS the wafer was covered with a solution of 10μg ml^{-1} rabbit IgG and exposed to light from a high pressure mercury vapour lamp for 15 minutes through a chrome mask patterned with grids having lines of width 2μm, 4μm, 6μm and 8μm in equal mark space ratio. After extensive rinsing with PBS the wafer was covered with a solution of 10mg ml^{-1} bovine serum albumin and was exposed to light from a high pressure mercury vapour lamp for 15 minutes.

(2) Assessment of protein patterning

The wafer was covered with a solution of 10μg ml^{-1} TRITC labelled goat anti-rabbit IgG, for 60 minutes at ambient temperature. After washing in PBS at distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy. Areas of fluorescence were observed which matched the mask that had been used. Features as small as 4μm could be resolved.

Example 4

(1) Light dependent coupling of two proteins to a silicon dioxide surface

Avidin D and photobiotin were immobilised onto the silicon dioxide surface as described in Example 3. After extensive rinsing with PBS the wafer was covered with a solution of 10μg ml^{-1} rabbit IgG and exposed to light from a high pressure mercury vapour lamp for 15 minutes through a chrome mask patterned with a 25μm grid in equal mark space ratio. After extensive rinsing with PBS the wafer was covered with a solution

of 10μg ml^{-1} rat IgG and exposed to light from a high pressure mercury vapour lamp for 15 minutes through the same mask used in Example 3 that had been turned through an angle of 90°. After extensive rinsing with PBS the wafer was covered with a solution of 10mg ml^{-1} bovine serum albumin and was exposed to light from a high pressure mercury vapour lamp for 15 minutes.

(2) Assessment of protein patterning

The wafer was covered with a solution of 10μg ml^{-1} TRITC labelled goat anti-rabbit IgG, for 60 minutes at ambient temperature. After extensive rinsing with PBS the wafer was covered with a solution of 10μg ml^{-1} FITC labelled rabbit anti-rat IgG, for 60 minutes at ambient temperature. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using a fluorescent microscope. Unbroken lines of red fluorescence corresponding to the immobilised rabbit IgG were observed, and lines of green fluorescence corresponding to the immobilised rat IgG were observed running perpendicular to the red lines. Where the fluorescent lines crossed the green lines due to rat IgG were discontinued.

Example 5

(1) Light dependent coupling of a protein to a glass surface

A glass wafer was immersed in a 1% solution of 1,3-trimethoxysilylpropylethylene diamine in 95% ethanol 5% distilled water, pH adjusted to 5.0 with glacial acetic acid for 30 seconds. After removing the wafer from this solution it was rinsed briefly in 95% ethanol 5%

distilled water before being heated at 120°C for 30 minutes. The wafer was immersed in a 2% solution of gluteraldehyde in PBS for 15 minutes, and then in a solution of 40mM sodium cyanoborohydride, 0.2mg ml^{-1} Avidin D in PBS for 30 minutes at ambient temperature. After extensive rinsing with PBS the wafer was then incubated in 5ml of a 10µg ml^{-1} solution of long arm photobiotin in PBS for 20 minutes under dark room conditions. After extensive rinsing with PBS the wafer was covered with a solution of 10µg ml^{-1} rat IgG and exposed to light from a high pressure mercury vapour lamp for 15 minutes through a patterned chrome mask. After extensive rinsing with PBS the wafer was covered with a solution of 10mg ml^{-1} bovine serum albumin and was exposed to light from a high pressure mercury vapour lamp for 15 minutes.

(2) Assessment of protein patterning

The wafer was covered with a solution of 10µg ml^{-1} FITC labelled anti-rat IgG, for 60 minutes at ambient temperature. After washing in PBS and distilled water the sample was dried in a stream of nitrogen and examined using fluorescent microscopy. Areas of fluorescence were observed which matched the mask that had been used.

Example 6

Cell Guidance

(1) Patterning of silicon surface

A wafer of silicon dioxide was immersed in a 1% solution of 1,3-trimethoxysilylpropyl- ethylene diamine

in 95% ethanol 5% distilled water for 120 seconds. After removing the wafer from this solution it was rinsed briefly in 95% ethanol 5% distilled water before being heated at 120°C for 30 minutes. The wafer was immersed in a 2% solution of gluteraldehyde in PBS for 15 minutes, and then in a solution of 40mM sodium cyanoborohydride, containing 0.2 mg ml^{-1} Avidin D in PBS for 30 minutes at ambient temperature. After extensive rinsing with PBS the wafer was then incubated in 5ml of a 10 μg ml^{-1} solution of long arm photobiotin in PBS for 20 minutes under dark room conditions. After extensive rinsing with PBS the wafer was covered with a sterile solution of 10 mg ml^{-1} concanavalin A and exposed to light from a high pressure mercury vapour lamp for 15 minutes through a chrome mask patterned with a 12.5 μm grid . After extensive rinsing with PBS the wafer was covered with a sterile solution of 10mg ml^{-1} bovine serum albumin and was exposed to light from a high pressure mercury vapour lamp for 15 minutes.

(2)

Snails were by placed in 25% Listerine for 5 minutes, and their brains were dissected out and incubated in 1 mg ml^{-1} Pronase at ambient temperature for 90 minutes. Individual cells were isolated and placed onto the patterned silica wafer in growth media consisting of 33% (v/v) Gibco L-15, but with the $CaCl_2$ and $MgCl_2$ concentrations adjusted to 5.5mM and 2.43 mM respectively. The growth media also had additions of 50 μg ml^{-1} gentamycin and 0.2% glucose (w/v). The cells were incubated at 20°C for 7 days.

(3) Assessment of cell guidance.

The cells were examined under a microscope. Cell processes were to seen to run parallel with each other, and the distance between the processes was consistent with the patterning of the protein on the silicon dioxide.

Example 7

Multianalyte Sensor

In this example a multianalyte immunosensor for the quantitative determination of the human gonadotrophin hormones (follicle stimulating hormone and luteinising hormone) is produced. The assay is based upon the electrochemical detection of two horseradish peroxidase labelled antibodies using a ferrocene mediated system. Results obtained with the biosensor showed a good correlation with those obtained using an established clinical diagnostic technique based upon dissociation-enhanced lanthanide fluorometric immunoassay.

EXPERIMENTAL

Electrode Fabrication

Sensor arrays were produced on 10 cm diameter silicon wafers. Immobilisation of proteins was performed before the wafer was cut into individual devices, so that the preparation of all arrays was identical. Gold electrodes were prepared using standard photolithographic procedures. Both the electrodes and bonding pads were exposed whilst all other areas were electrically insulated using hardened photoresist. Ag/AgCl reference electrodes were prepared by electrosorbtion of silver onto specified gold

electrodes from a solution of 0.1M $AgNO_3$ in 0.1M sulphuric acid with a silver anode (at a constant current of 0.4mA cm^{-2} for 6 hours) followed by chloridisation in 0.1M HCl (0.4 mA cm^{-2} for 30 minutes). The electrochemical behaviour of the fabricated electrodes was verified using cyclic voltammetry (-0.2 to +0.75 V scanned at 20 mV s^{-1}) in 0.2 mM ferrocene monocarboxylic acid (Sigma, Poole, England) containing 50 mM Tris 50 mM KCl, pH 7.4. Results were compared with those obtained using a Bioanalytical Systems (BAS) gold working electrode and a BAS RE4 Ag/AgCl reference electrode (Biotech Instruments Ltd, Luton, England). Reproducibility of electrode arrays prepared in this manner, was assessed by measuring the chronoamperometric response (10 seconds at 0V, 120 seconds at +650 mV) in the presence of 0.5 mM H_2O_2 in 50 mM sodium phosphate buffer containing 50 mM KCl, pH 7.4. All experiments involving the fabrication and characterisation of electrodes were performed using an EG&G 273A potentiostat (EG&G, Sunninghill, England).

Antibody Immobilisation

The immobilisation procedure is outlined in Figure 3. Neutravidin™, a modified form of avidin (Pierce and Warriner, Chester, UK) was attached to the gold electrode surface using activation of a self-assembled thiol monolayer (in this case N-acetyl-l-cysteine (Sigma)) with a water soluble carbodiimide. Electrode arrays were first incubated in 2 mM N-acetyl-l-cysteine in 10 mM phosphate buffer (pH 7.0) for 120 minutes at ambient temperature, followed by 120 minutes incubation in 1% (w/v) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma) in 10 mM phosphate buffer (pH 7.0). The modified gold sensor arrays were then

incubated in 100Ag ml^{-1} Neutravidin in 10 mM phosphate buffer (pH 7.0) for 16 hours at 4°C. All subsequent stages of the immobilisation procedure were performed at ambient temperature. After washing in phosphate buffered saline (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCI), pH 7.4 (PBS), the electrodes were incubated first in 10 mg ml^{-1} casein in PBS for 60 minutes and then in 10 µg ml^{-1} long arm photobiotin (Vector Laboratories, Peterborough, England) in PBS, for 20 minutes in the dark. All subsequent immobilisation stages were performed in a dark room. After washing in PBS, the wafer was covered with 10 Ag ml^{-1} monoclonal anti-FSH (Biogenesis Ltd, Bournemouth, England, clone BIO-FSHB-003), and selected electrodes were exposed to light from a 100W HG-10101AF super high pressure mercury vapour lamp (Nikon, Tokyo, Japan) 185 mm from the electrodes for 15 minutes (Irradiance = 9 mW cm^{-2}) using a suitable mask. It is important to note that light of wavelengths below 300 nm was removed by passing through a glass filter to prevent denaturation of proteins.

After washing in PBS, the wafer was covered with 10 µg ml^{-1} monoclonal anti-LH (Biogenesis clone LH-007), and selected electrodes were exposed to light from the lamp for 15 minutes, prior to washing in PBS. The entire wafer was exposed to light from the lamp for 15 minutes in the presence of 10 mg ml-1 casein in PBS, and washed in PBS.

Immumoassay procedure

The immunoassay, which was an enzyme linked immunosorbent assay (ELISA) based upon a "sandwich" format, was configured with immobilised "capture"

antibodies on the electrode surfaces such that the addition of a second enzyme labelled antibody was directed against a second epitopic site on the antigen. Sensors were incubated with 250 µl of sample for 60 minutes, washed thoroughly with PBS, and incubated in a mixture of 10 µg ml^{-1} horseradish peroxidase (HRP) labelled anti-LH (Biogenesis clone BIO-FSHB-002) and 10 µg ml^{-1} HRP labelled anti-LH (Biogenesis clone LH-005) in PBS for 60 minutes at ambient temperature, before, finally, being washed in PBS. Simultaneous assessment of HRP activity at the FSH and LH sensor electrodes was performed chronoamperometrically using two Bioanalytical System CV-37 potentiostats (Biotech Instruments Ltd, Luton, England) and a Goerz SE120 dual channel chart recorder (Belmont Instruments, Glasgow, UK). Activity was determined at +150 mV vs Ag/AgCl by measuring the current produced after 20 seconds in the presence of 10 mM hydrogen peroxide and 0.2 mM ferrocene monocarboxylic acid in 50 mM phosphate buffer containing 50 mM KCl, pH 7.4. The response of the immunosensor to hormone concentration in a buffered aqueous solution was measured by preparation of a series of standards (0 - 100 UI^{-1}) of FSH and LH (Biogenesis) which covered the concentration range of clinical interest. The results obtained were subsequently used to construct a calibration curve for further experiments.

The multi-analyte immunosensor was used to determine gonadotrophin concentrations in 10 serum samples from hospital outpatients. The analyses were performed on three separate occasions using a newly constructed calibration curve each time. The results obtained were compared with those obtained using an established DELFIA technique (see Lovgren et al Talanta 31: 909-916

(1984)). The samples examined covered the range of values typically seen in clinical laboratories.

RESULTS AND DISCUSSION

Patterning of Antibodies on Electrode Surfaces

Central to designing a multianalyte immunosensor is overcoming the problem of patterning of antibodies at discrete locations without encountering high levels of non-specific binding. In this example this difficulty has been overcome by using biological self-assembly of avidin and a biotin derivative, called photobiotin. The first stage of the patterning technique therefore involves immobilising either avidin or its microbial counterpart streptavidin onto a surface. Both of these are tetrameric proteins that specifically bind biotin with an association constant of 10^{15} M^{-1}. Photobiotin is bound to the avidin-modified surface to provide a light sensitive "addressable" surface onto which molecules can be "written" using an appropriate light source and a mask. Photobiotin contains an aryl azide group which is stable in the dark, but which, upon exposure to light (340-375 nm) forms a highly reactive aryl nitrene group. This will bind organic species present in the solution above the surface by a number of mechanisms including insertion into C-H or N-H bonds, and addition to C=C bonds. After immobilisation of the avidin, the surface was exposed to a solution of photobiotin which bound to the avidin-modified surface (Fig. 3a). Exposure of selected areas of this surface to light resulted in activation of the photobiotin molecule (Fig. 3b), so that antibodies present in the solution were immobilised onto the surface. To minimise the problem of non-specific binding of proteins at the

avidin modified surface, a modified form of avidin (Neutravidin) which has low non-specific binding characteristics was used. Consequently, few protein molecules adhere to the surface non-specifically compared with the number that are bound by activated photobiotin. Any unbound material can be removed by washing. The patterning procedure can be repeated sequentially with a second protein (Fig. 3c) or with any number of proteins thereafter. In order to ensure that all unreacted photobiotin groups are "neutralised", the entire surface is exposed to light in the presence of a blocking molecule (eg casein or bovine serum albumin) (Fig. 3d).

Characterisation of Electrodes

The potentials at which oxidation and reduction peaks were evident upon cyclic voltammetry of ferrocene monocarboxylic acid for the fabricated electrodes were within 5 mV of those obtained when using standard BAS working and reference electrodes (E_{pa} = 355 mV, E_{pc} = 296 mV). The intra-batch coefficient of variation for the responses of the electrode arrays to 0.5 mM H_2O_2 was 1.86% (n=20), whilst the interbatch coefficient of variation was 2.43% (n=5).

Immunosensor Response

The response of the sensor to FSH and LH in buffer was measured over the range 0 to 100 UI^{-1}, Figures 4 and 5. When corrected for the specific activities of the hormone preparations, these ranges are equivalent to 0 to 26 ng l^{-1} and 0 to 18 ng l^{-1} for FSH and LH respectively. Figure 4 demonstrates that the current at the FSH sensor is proportional to the FSH

concentration (2.1 nA /Ul^{-1} (8.0 nA/ngl^{-1})), and that the response of the LH sensor to FSH is negligible (0.07 nA/Ul-1 (0.38 nA/ngl^{-1})). Likewise, Figure 5 demonstrates that the current produced by the LH sensor is proportional to the LH concentration (2.5 nA / Ul-1 (13.6 nA /ng l^{-1})), and the response of the FSH sensor to LH is negligible (0.11 nA / Ul^{-1} (0.42 nA / ngl^{-1})).

The response when no antigen is present is due to a number of factors, chief amongst these is the current resulting from electrochemical processes unrelated to the immunoassay (ie the background current obtained when there is no enzymic activity). The remainder of the current measured, when the antigen concentration is zero, is due either to non-specific binding or to diffusion of electroactive species between electrodes.

Of the several causes of non-specific binding, the binding of an inappropriate antibody at a sensor site (eg anti-LH on a sensor for FSH) is of particular importance in a multianalyte immunosensor. This can occur for a number of reasons, such as binding through non-specific protein-protein interactions, hydrophobic interactions with non-polar surfaces, or electrostatic interactions between the protein and the surface, and results in an inappropriate antibody being able to bind its complimentary antigen and the enzyme labelled second antibody.

Figures 6 and 7 show results for human serum samples obtained from the multianalyte sensor compared with those from an established DELFIA technique. This latter method uses lanthanides (which have a relatively long lived fluorescence) such as europium as fluorescent labels in immunoassays. The intensity of

the fluorescence is enhanced by dissociating the label from the immunocomplex prior to measurement. There is a very good correlation between the two methods, and close agreement between results at all concentrations for both FSH ($[FSH]_{IMMUNOSENSOR} = 0.9756\ [FSH]_{DELFIA} + 0.332$, $r^2 = 0.9990$) and, LH ($[LH]_{IMMUNOSENSOR} = 0.9815\ [LH]_{DELFIA} + 0.125$, $r^2 = 0.9996$).

Conclusion

The immobilisation procedure described enables the selective and specific patterning of multiple functional proteins with minimal non-specific binding. The process has the potential to be miniaturised with micrometre resolution and therefore may be used to produce multianalyte microsensors.

The applicability of this technique to multianalyte immunoassays has been demonstrated using determination of gonadotrophins as a model system. Although a sensor for measuring two analytes has been constructed, the technology that has been developed is compatible with the fabrication f a sensor for a greater number of analytes. The fabrication and immobilisation procedures used in this work would be compatible with manufacturing technology commonplace in the microelectronics industry. Additionally, there is no waste of expensive proteins such as monoclonal antibodies as non-immobilised excess protein can easily be recovered, and be reused.

Example 8

Patterning of Nucleic Acids

A SiO_2 wafer was immersed in 1% 1,3-trimethoxysilyl-propylethylene diamine in 95:5 (v/v) ethanol/distilled water for 120 seconds and briefly rinsed in 95:5 (v/v) ethanol/distilled water before heating at 120°C for 30 minutes. The wafer was immersed in 2% gluteraldehyde in phosphate buffered saline (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS)) for 15 minutes, and in 40 mM sodium cyanoborohydride, 0.2mg ml^{-1} Neutravidin™ (Pierce & Warriner, Chester, UK) in PBS for 30 minutes. The SiO_2 substrate was washed in PBS after this and all subsequent steps. The avidin-modified wafer was incubated in 5 ml of 10 μg ml^{-1} long arm photobiotin (Vector) in PBS for 20 minutes, this and all subsequent stages were performed under dark room conditions.

A solution of biotinylated DNA in PBS, was layered on to the SiO_2 wafer and a photolithographic mask with 3 μm lines (equal mark-space ratio) was placed on top. The sample was then exposed to light from a 100W high pressure mercury vapour lamp for 15 minutes (irradiance = 9 mW cm^{-2}). Following exposure, the mask was removed and it and the wafer were thoroughly washed with PBS.

The sample was incubated in fluorescein isothiocyanate (FITC) labelled avidin for 2 hours, dried under a gentle stream of nitrogen and examined using fluorescence microscopy. A pattern corresponding to that of the photolithographic mask was observed.

Modifications and variations of the above described embodiments can be adopted without departing from the scope of the invention.

Claims

1. A device having a surface, said surface having a ligand bound thereto in a pre-determined pattern, the binding of said ligand being determined by the irradiation or non-irradiation of a photosensitive binding moiety attached to said surface via a linking moiety.

2. A device as claimed in Claim 1 wherein two or more ligands are bound to said surface.

3. A device as claimed in either one of Claims 1 and 2 which produces a measureable change.

4. A device as claimed in any one of Claims 1 to 3 wherein said linking moiety is avidin or a functional equivalent thereof.

5. A device as claimed in any one of Claims 1 to 4 wherein said photosensitive binding moiety is photobiotin or a functional derivative thereof.

6. A device as claimed in any one of Claims 1 to 5 wherein said ligand is a hormone, an enzyme or an immunoglobulin.

7. A device as claimed in any one of Claims 1 to 6 wherein substantially all of the photosensitive binding moiety not bound to ligand is bound to a blocking protein.

8. A device as claimed in any one of Claims 1 to 7 wherein the surface is silicon, silicon nitride, silicon dioxide, glass, quartz, metals, metal

oxides, polymers and/or mixtures thereof.

9. A device as claimed in any one of Claims 1 to 8 for use in a multi-analyte sensor, in molecular electronics, in binding nucleotides, in directional propagation of cells, and/or in alteration of cell behaviour.

10. Use of a device as claimed in any one of Claims 1 to 8 in a multi-analyte sensor.

11. Use as claimed in Claim 10 in a multi-analyte immunosensor.

12. Use of a device as claimed in any one of Claims 1 to 8 in molecular electronics.

13. Use of a device as claimed in any one of Claims 1 to 8 in directional propagation of cells.

14. Use of a device as claimed in any one of Claims 1 to 8 in the alteration of cell behaviour.

15. An immunosensor comprising a device as claimed in any one of Claims 1 to 8.

16. A multi-analyte immunosensor comprising a device as claimed in any one of Claims 1 to 8.

17. An electronics device comprising a device as claimed in any one of Claims 1 to 8.

18. Cells obtained by propagation using a device as claimed in any one of Claims 1 to 8.

19. A process for forming a surface having a ligand bound thereto in a pre-determined pattern, said process comprising the following steps:

i) binding a linking moiety to a coated or uncoated surface;

ii) binding a photosensitive binding moiety to said linking moiety;

iii) selectively exposing said photosensitive binding moiety to irradiation in a pre-determined pattern;

iv) exposing said binding moiety to said ligand and allowing the ligand to bind to said binding moiety in accordance with the irradiation exposure;

v) optionally removing excess ligand by washing; and

vi) optionally exposing said ligand to a further molecule capable of binding thereto.

20. A process as claimed in Claim 19 wherein steps iii) and iv) are repeated at least once to bind a second ligand to said surface in a pre-determined pattern.

21. A process as claimed in either one of Claims 19 and 20 wherein substantially all of the photosensitive binding moiety not bound to ligand is subsequently bound to a blocking moiety.

22. A process as claimed in any one of Claims 19 to 21 wherein selective irradiation or non-irradiation of said photosensitive binding moiety is achieved

by use of a mask.

23. Use of a device as claimed in any one of Claims 1 to 8 for diagnosis.

24. Use of a device as claimed in any one of Claims 1 to 8 for binding nucleotides.

Figure 1

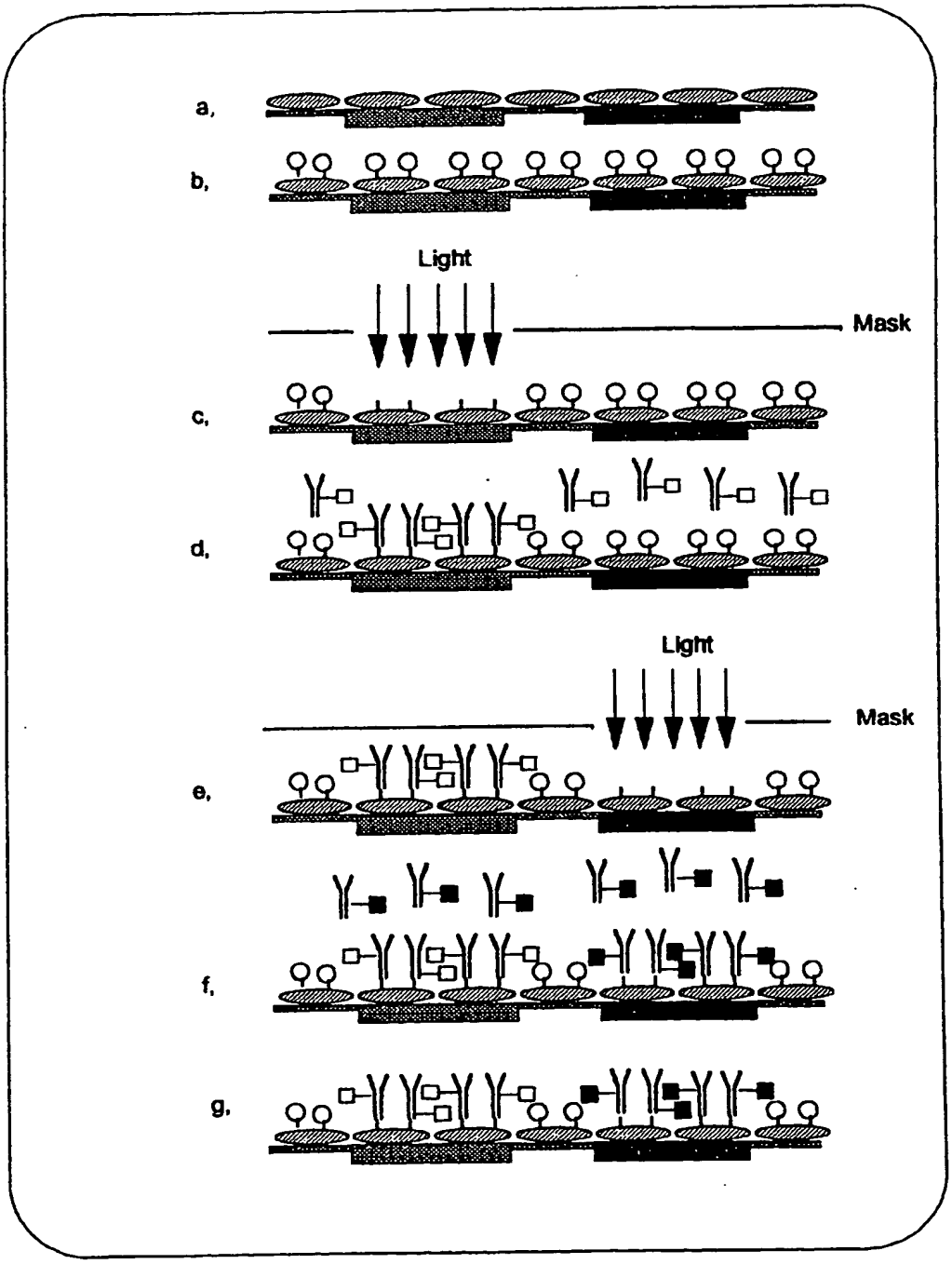


Figure 2

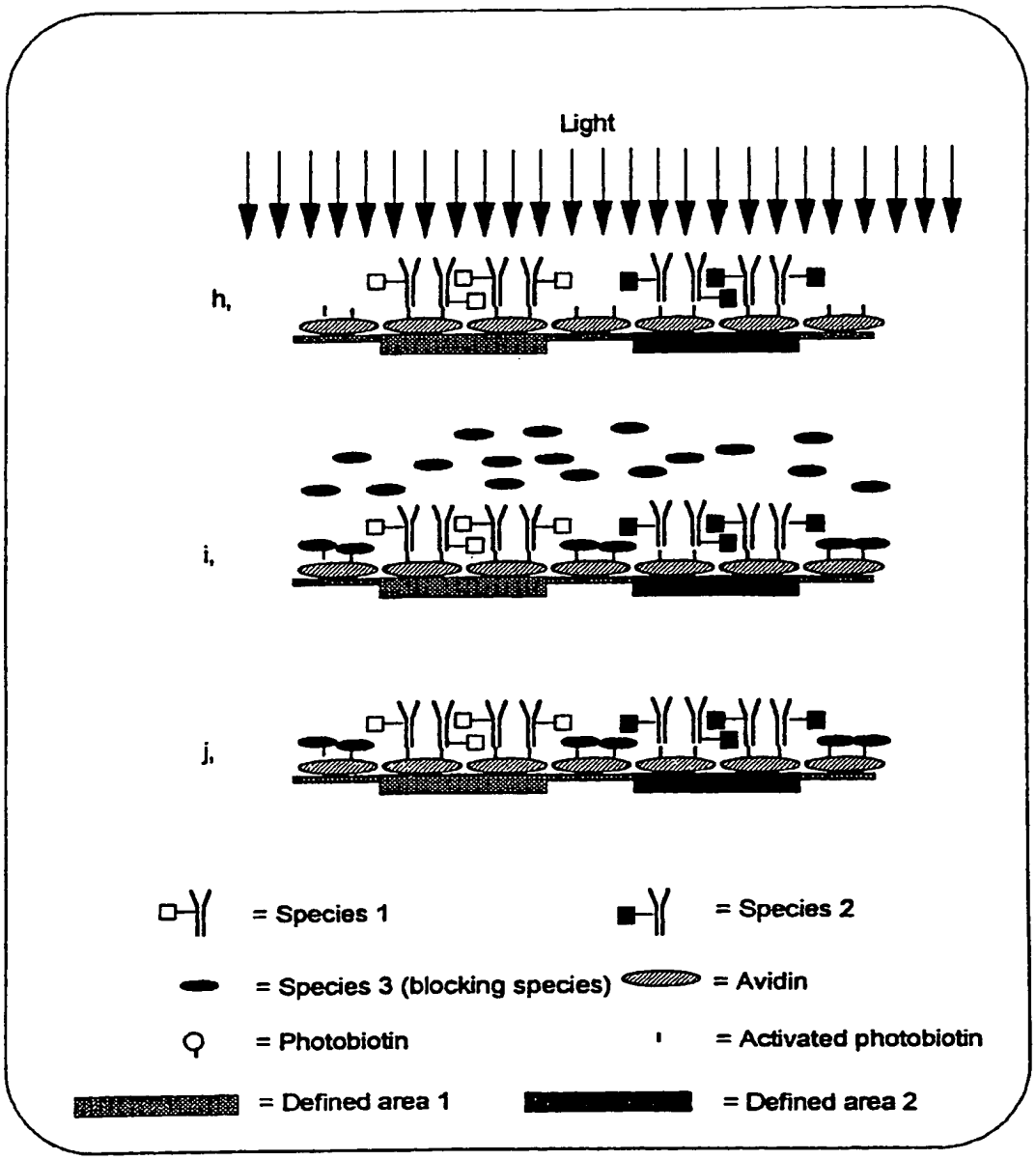

Light
h,
i,
j,
= Species 1
= Species 2
= Species 3 (blocking species)
= Avidin
= Photobiotin
= Activated photobiotin
= Defined area 1
= Defined area 2

Figure 3

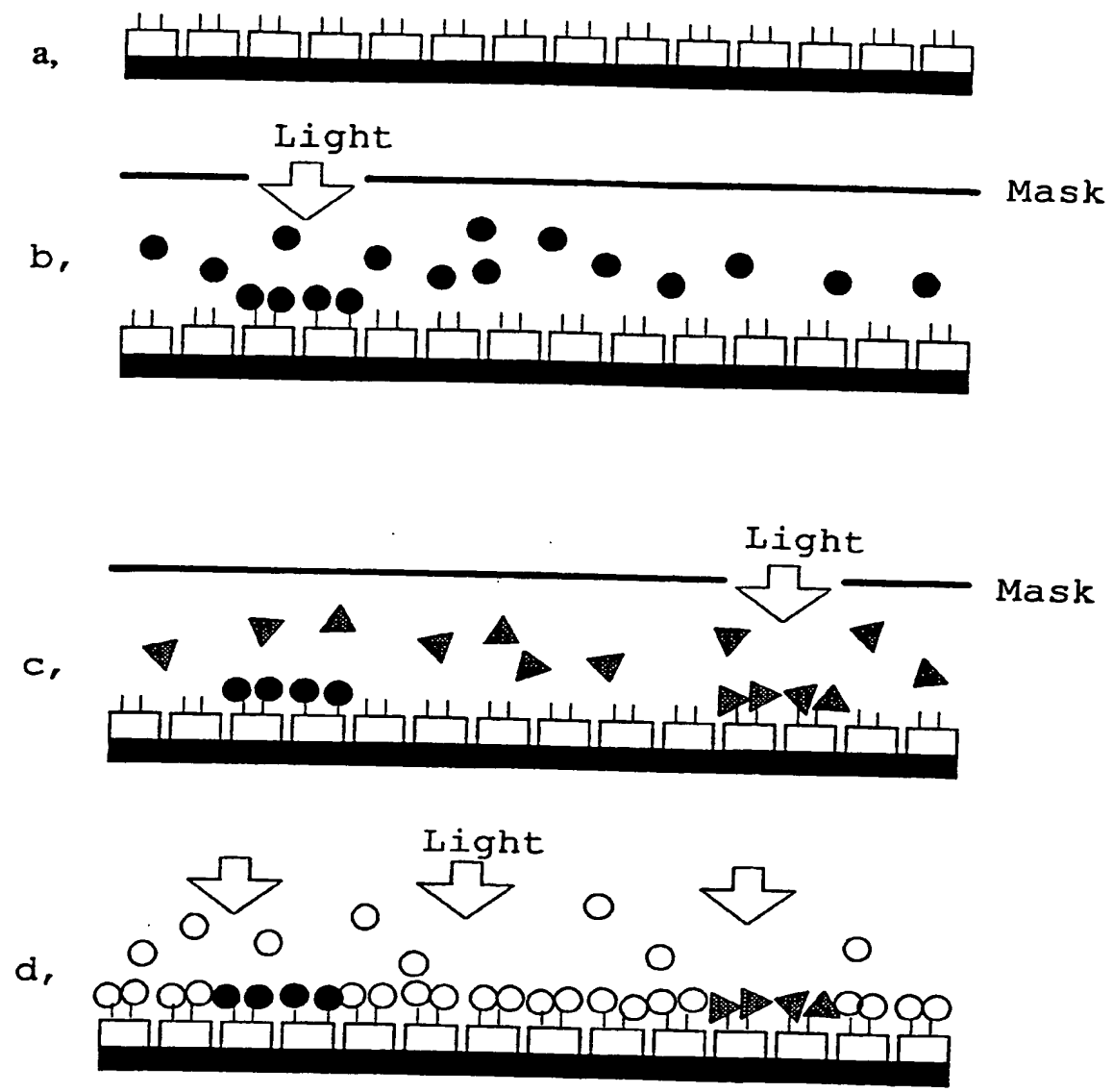
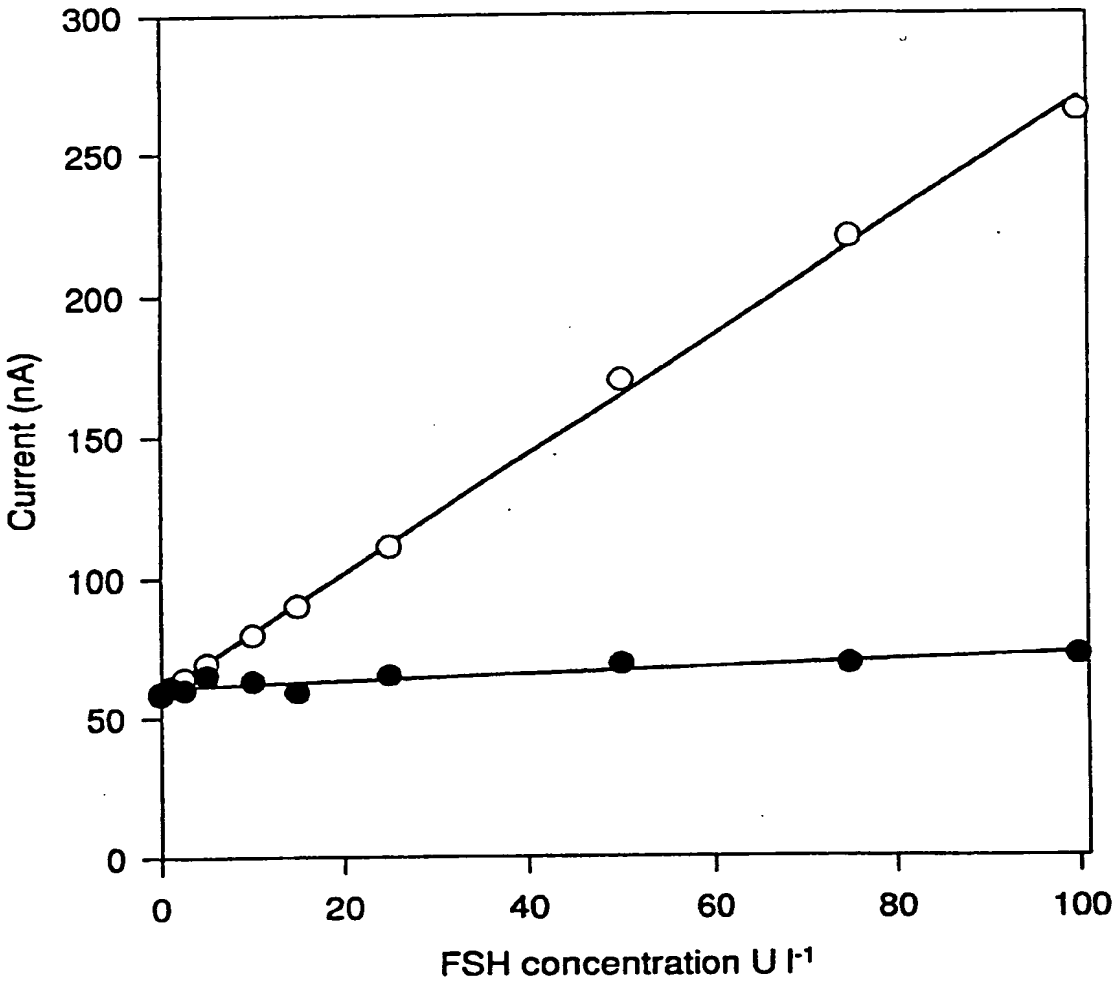

a,
Light
Mask
b,
Light
Mask
c,
Light
d,
= avidin
= photobiotin
= 1st protein
= 2nd protein
= blocking species

Figure 4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Current (nA)
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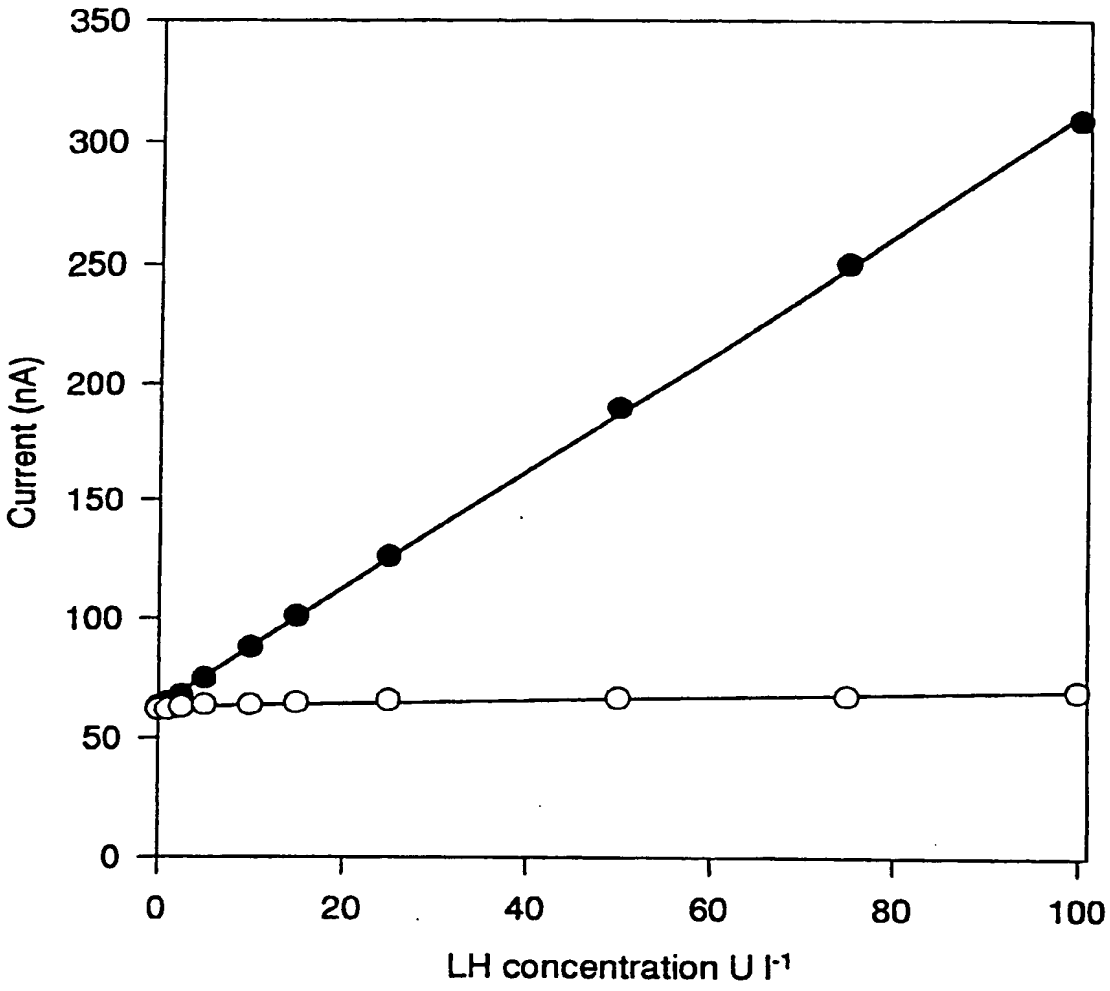
40
60
80
100
FSH concentration U l-1

Figure 5


350
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150
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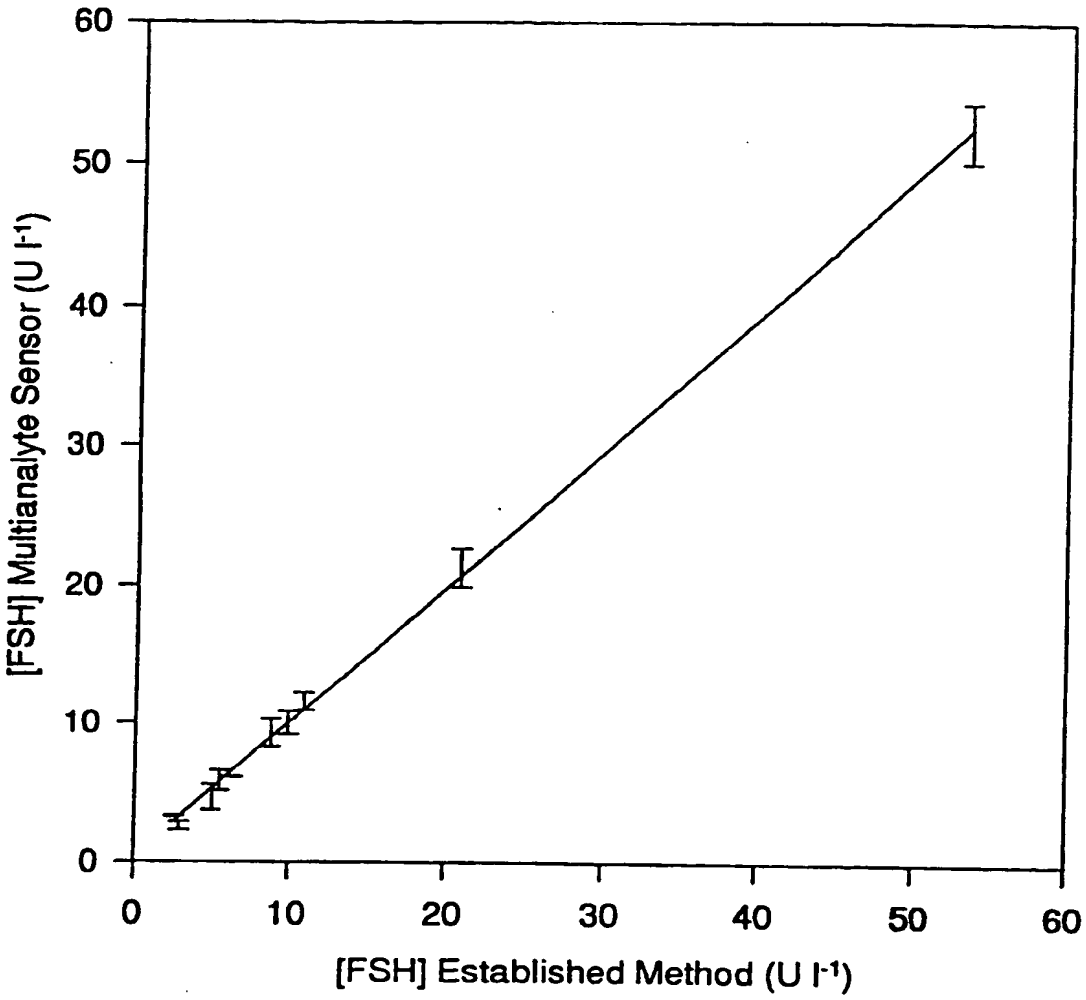
50
0
Current (nA)
0
20
40
60
80
100
LH concentration U l-1

Figure 6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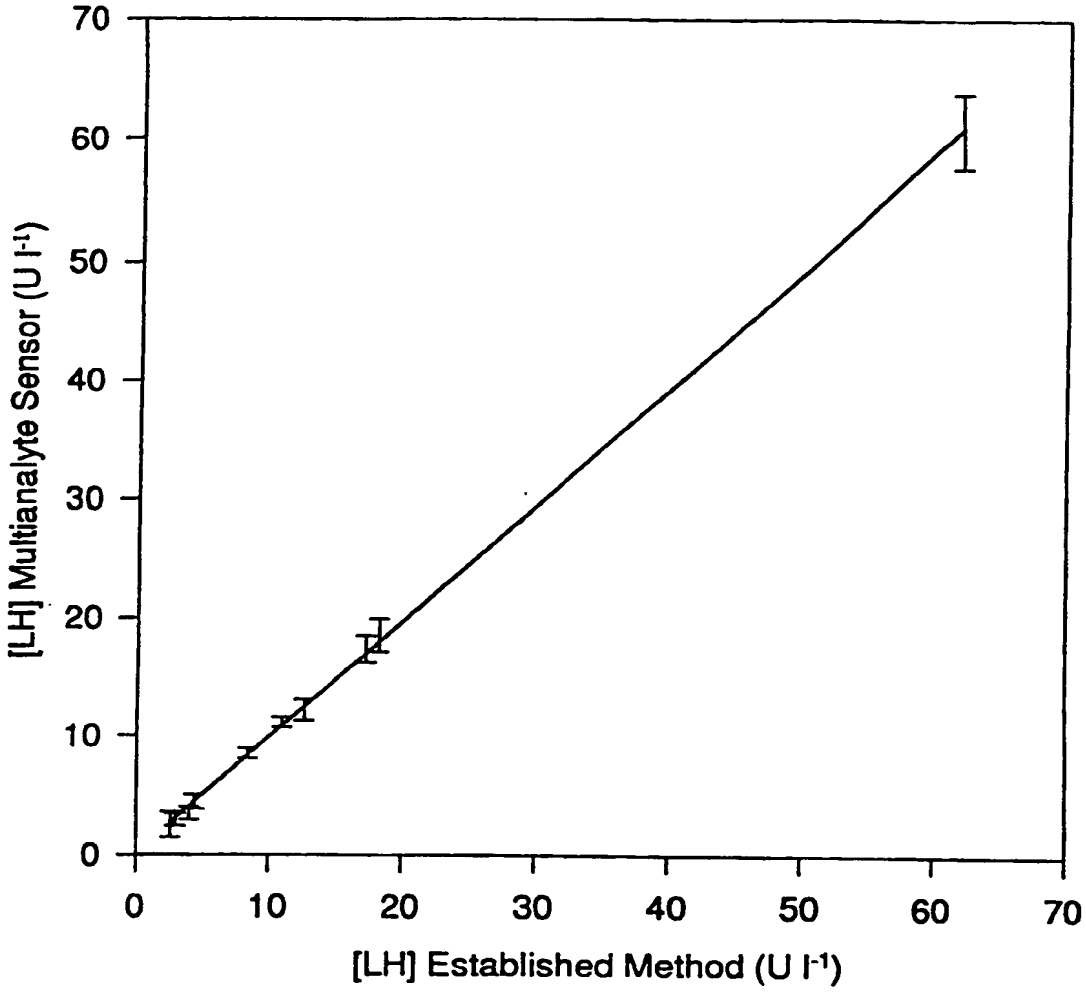
[FSH] Multianalyte Sensor (U l-1)
[FSH] Established Method (U l-1)

Figure 7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30
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10
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[LH] Multianalyte Sensor (U l-1)
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30
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50
60
70
[LH] Established Method (U l-1)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 94/02680

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 G01N33/547 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF IMMUNOLOGICAL METHODS., vol.132, 1990, NEW YORK US pages 91 - 101 W. EMLEN ET AL. 'A NEW ELISA FOR THE DETECTION OF DOUBLE-STRANDED DNA ANTIBODIES.' see the whole document ---	1-24
Y	EP,A,0 127 438 (NATIONAL RESEARCH DEVELOPMENT CO.) 5 December 1984 cited in the application see the whole document & GB,A,2 141 544 ---	1-24
A	WO,A,91 07087 (AFFYMAX TECHNOLOGIES, N. V.) 30 May 1991 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 April 1995	10. 05. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Cartagena y Abella,P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEIC ACIDS RESEARCH, vol.13, no.3, 1985, ARLINGTON, VIRGINIA US pages 745 - 761 A.C. FORSTER ET AL. 'NON-RADIOACTIVE HYBRIDIZATION PROBES PREPARED BY THE CHEMICAL LABELLING PF DNA AND RNA WITH A NOVEL REAGENT, PHOTOBIOTIN.'	

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 94/02680

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0127438	05-12-84	CA-A-	1229883	01-12-87
		DE-A-	3468146	28-01-88
		GB-A,B	2141544	19-12-84
		JP-B-	6050316	29-06-94
		JP-A-	59225343	18-12-84
		US-A-	4562157	31-12-85
GB-A-2141544	19-12-84	CA-A-	1229883	01-12-87
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		JP-T-	5501611	25-03-93
		US-A-	5252743	12-10-93